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P2Y receptor-mediated Ca^{2+} signalling in cultured rat aortic smooth muscle cells

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1 ATP, UTP, ADP and ADP-β-S elicited Ca^{2+}-signals in cultured aortic smooth muscle cells although ADP, UDP and ADP-β-S gave $\sim$40% of the maximal response seen with ATP and UTP. Adenosine, AMP or α,β-methylene-ATP had no effect. These responses were attributed to $P2Y_{2/4}$ and $P2Y_1$ receptors, which we assumed could be selectively activated by UTP and ADP-β-S respectively.

2 The response to UTP was reduced ($\sim$50%) by pertussis toxin, whilst this toxin had no effect upon the response to ADP-β-S. This suggests $P2Y_{2/4}$ receptors simultaneously couple to pertussis toxin-sensitive and -resistant G proteins whilst $P2Y_1$ receptors couple to only the toxin-resistant proteins.

3 Repeated stimulation with UTP or ADP-β-S caused desensitization which was potentiated by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and attenuated by staurosporine.

4 TPA completely abolished sensitivity to ADP-β-S but the response to UTP had a TPA-resistant component. In pertussis toxin-treated cells, however, TPA could completely abolish sensitivity to UTP and so the TPA-resistant part of this response seems to be mediated by pertussis toxin-sensitive G proteins.

5 Loss of sensitivity to UTP did not occur when pertussis toxin-treated cells were repeatedly stimulated with this nucleotide, suggesting that pertussis toxin-sensitive G proteins mediate this effect. The toxin did not, however affect desensitization to ADP-β-S.

Keywords: P2Y-receptor; Ca^{2+} mobilization; pertussis toxin; receptor desensitization; protein kinase C; aortic smooth muscle; cell culture

Abbreviations: α,β-me-ATP, α,β methylene ATP; ADP-β-S, adenosine; AMP, adenosine monophate; EDTA, ethylenediamine tetra-acetic acid; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; UDP, uridine diphosphate; UTP, uridine triphosphate

Introduction

Extracellular nucleotides elicit complex responses in essentially all components of the vascular system (reviewed by Burnstock, 1998) and our preliminary studies of cultured aortic smooth muscle cells (Pediani *et al*., 1995) showed that ATP and UTP increased intracellular free Ca^{2+} ($[Ca^{2+}]_i$) and that this response was initiated by the mobilization of Ca^{2+} from a cytoplasmic store (see also Droogmans *et al*., 1991; Kalthof *et al*., 1993). The response thus appears to be mediated *via* P2Y receptors, which characteristically allow nucleotides to activate a phosphatidyl inositol specific isoform of phospholipase C (PI-PLC) (see e.g. Droogmans *et al*., 1991; Kalthof *et al*., 1993; Purkiss *et al*., 1993; Boarder *et al*., 1995; Nicholas *et al*., 1996; Boarder & Hourani, 1998; Harper *et al*., 1998). This signalling pathway forms a crucially important mechanism by which extracellular hormones and neurotransmitters are able to control intracellular events (Berridge, 1993) and so, in the present study, we have sought to characterize the receptors/signalling pathways that underlie these nucleotide-evoked $[Ca^{2+}]_i$-signals. Accounts of the work have been presented to The Physiological Society (Pediani *et al*., 1996; 1997).

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Methods

Solutions and chemicals

The physiological salt solution contained (in mM): NaCl, 130; KCl, 5; $CaCl_2$, 1; $MgCl_2$ 1; 4-(2-hydroxyethyl)-1-piperazine sulphonic acid (HEPES), 20; D-glucose, 10; pH adjusted to 7.4 using NaOH. Ca^{2+}-free solutions were prepared by simply omitting $CaCl_2$ from the control solution. The culture medium was Medium 199 supplemented with foetal calf serum (FCS, 10% vol vol^{-1}), penicillin (100 i.u. ml^{-1})/streptomycin (100 $\mu g\ ml^{-1}$), bovine insulin (10 $\mu g\ ml^{-1}$), L-glutamine (1 mM) and sodium pyruvate (1 mM). The digestion medium was Medium 199 containing collagenase (1 mg ml^{-1}), elastase (0.1 mg ml^{-1}), soyabean trypsin inhibitor (0.5 mg ml^{-1}), bovine serum albumen (BSA, 1 mg ml^{-1}) and penicillin (200 $\mu U\ ml^{-1}$)/streptomycin (200 $\mu g\ ml^{-1}$). FCS, sodium pyruvate, L-glutamine, bovine insulin, antibiotics and trypsin/ethylenediamine tetra-acetic acid (EDTA) were from Gibco Life Technologies (Paisley, U.K.). Fura 2-AM, uridine triphosphate (UTP), adenosine triphosphate (ATP), adenosine diphosphate (ADP), uridine diphosphate (UDP), adenosine monophate (AMP), adenosine, ADP-β-S, α,β-methylene-ATP (α,β-me-ATP), elastase, collagenase, BSA, soyabean trypsin inhibitor, HEPES, staurosporine, 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and pertussis toxin were from Sigma (Dorset, U.K.). All ADP and UDP solutions used were prepared from stock solutions (10 mM) that had been pre-treated with hexokinase (Boehringer, Mannhein, Germany) in the presence of 22 mM D-Glucose to ensure that contaminating nucleotide triphosphates were converted to the respective diphosphates

(see Harden *et al*., 1997). Stock solutions of TPA (1.62 mM in dimethyl sulphoxide) and staurosporine (1 mM in methanol) were prepared, aliquoted and stored at −20°C. Solutions containing these substances were freshly prepared on each experimental day.

Cell culture

Male Wistar rats were killed by cervical dislocation and, under aseptic conditions, their thoracic aortas were removed, rinsed in Medium 199 containing penicillin (200 μU ml^{-1})/streptomycin (200 μg ml^{-1}) and dissected free of fat and connective tissue. The cleaned vessels from eight animals were incubated (30°C) in digestion medium for ~30 min and the tunicae adventitia then removed as an everted tube. The remaining tissue was rinsed with antibiotic-containing Medium 199, placed in 5 ml of fresh digestion medium and incubated for a further ~45 min at 37°C. The partly digested tissue was then washed with Medium 199, minced with scissors and incubated for a further 2.5 h in 8 ml of fresh digestion medium. Single cells were then prepared from this digest by tituration, washed twice by resuspension in culture medium and seeded into 25 cm^2 culture flasks at a density of 1.7×10^5 cells cm^{-2}. These cells were maintained at 37°C in water saturated air containing 5% CO_2, and passaged (1:3 split) weekly using trypsin/EDTA. For experiments, cells removed from the culture flasks were plated onto glass coverslips and incubated for 1–2 days before being used in experiments. Pertussis toxin-treated cells were incubated in culture medium containing this toxin (10 ng ml^{-1}) for 18–24 h before being used in experiments. Experiments were undertaken using cells between passages 3 and 20.

Quantification of nucleotide-evoked $[Ca^{2+}]_i$ signals

Cells on coverslips were loaded with the Ca^{2+}-sensitive dye Fura-2 by incubation (15–30 min, 37°C) in culture medium containing the dye's membrane-permeant acetoxymethylester form (1.5 μM). A rise in $[Ca^{2+}]_i$ causes a corresponding rise in the Fura-2 fluorescence ratio recorded from cells loaded with this dye, and this allows receptor mediated changes in $[Ca^{2+}]_i$ to be monitored using standard, microspectrofluorimetric techniques (Grynkiewicz *et al*., 1985). In the present study, coverslips bearing Fura-2 loaded cells were mounted in a heated chamber attached to the stage of a Nikon Diaphot inverted microscope where the cells superfused (37°C, 5 ml min^{-1}) with physiological saline. Fura-2 fluorescence ratios were recorded (excitation wavelengths 340 and 380 nm) at 1 Hz from groups of 1–5 cells. Data were digitized and recorded directly to computer disk using an interface and associated software (Version 5.2) obtained from Cairn Research (Faversham, Kent, U.K.).

Data analysis

Nucleotide-evoked $[Ca^{2+}]_i$-signals were quantified by measuring the fluorescence ratio at the peak of each response and subtracting from it the ratio measured immediately prior to stimulation. Pooled data are presented as mean ± s.e.mean, and values of n refer to the number of experiments in each group. The significance of any differences between means were evaluated using Student's t-test. To assess their relative sensitivity to nucleotides, cells were stimulated with a series of 30 s pulses of increasing concentrations (0.1 μM – 10 mM) of nucleotides that were delivered at intervals of at least 6 min. In experiments involving nucleotides other than ATP, the response to a 30 s pulse of this nucleotide (100 μM) was also measured so all data could be expressed as fractions of the response evoked by this standard stimulus. EC_{50} values were calculated from sigmoid curves fitted to the pooled data using a least square regression procedure implemented in a commercially available software package (Grafit 4.06, Erithacus Software, Staines, U.K.). In experiments that explored the extent to which cells lost sensitivity to nucleotides during repeated stimulation, cells were repeatedly stimulated with 1 min pulses of 100 μM UTP or ADP-β-s that were delivered at intervals of 6 min. Preliminary analyses showed that, in control cells, each successive stimulus elicited a smaller response and that the rate at which the cells lost sensitivity to the stimulating nucleotide could be described by the kinetic model defined by a single exponent. The results of these experiments were therefore analysed by fitting (Grafit 4.06) such single exponents to the data obtained during the individual experiments. This analysis allowed us to calculate half times ($t_{½}$) for the loss of sensitivity seen under the different experimental conditions. The statistical significance of any differences between these values was then evaluated using Student's unpaired t-test.

Results

Nucleotide-evoked $[Ca^{2+}]_i$ signals

ATP, UTP (Figure 1a), ADP, ADP-β-S (Figure 1b) and UDP consistently ($n>4$ for each) increased $[Ca^{2+}]_i$ in the cultured aortic smooth muscle cells. The responses to all nucleotides were qualitatively indistinguishable, consisting of a rapid rise to a peak value that was followed by rapid decline to a plateau phase that was sustained until the stimulating nucleotide was withdrawn. In some instances oscillations in $[Ca^{2+}]_i$ were seen during exposure to the nucleotides (Figure 1). Adenosine (1 and 3 mM), α,β-me-ATP (30 and 60 μM) and AMP (1 and 3 mM) failed to elicit discernible changes in $[Ca^{2+}]_i$ although each group of cells in which these compounds were tested ($n>5$ for each) responded to 100 μM ATP. The responses to ATP, UTP, ADP, ADP-β-S and UDP were concentration-dependent and the EC_{50} values were estimated to be 10.8 ± 0.2, 4.7 ± 0.8, 6.5 ± 1.4, 5.5 ± 0.9 and 140 ± 5 μM respectively (Figure 2). It was, however, clear that the maximal responses evoked by ADP (47.9 ± 4.1%), ADP-β-S (34.0 ± 3.4%) and UDP (41.6 ± 1.8%) were smaller than the maximal responses to ATP or UTP (Figure 2).

Figure 3 shows that the cells also responded to UTP and ADP-β-S (both 100 μM) in the absence of external Ca^{2+} but that the responses were not sustained under these conditions. Clear oscillations in $[Ca^{2+}]_i$ were seen in some instances although, even in the cells displaying this oscillatory response, $[Ca^{2+}]_i$ always returned to a stable, basal value after 2–3 min exposure to agonist. Figure 3 also shows that subsequently raising the external Ca^{2+} concentration to 1 mM, in the continued presence of agonist, elicited a second, sustained rise in $[Ca^{2+}]_i$ (Figure 2) and separate experiments ($n=3$) showed that simply withdrawing and replacing external Ca^{2+} did not elicit any change in $[Ca^{2+}]_i$. The responses to these nucleotides (Figure 1) thus appear to involve both the release of Ca^{2+} from a cytoplasmic store and receptor-regulated Ca^{2+} influx.

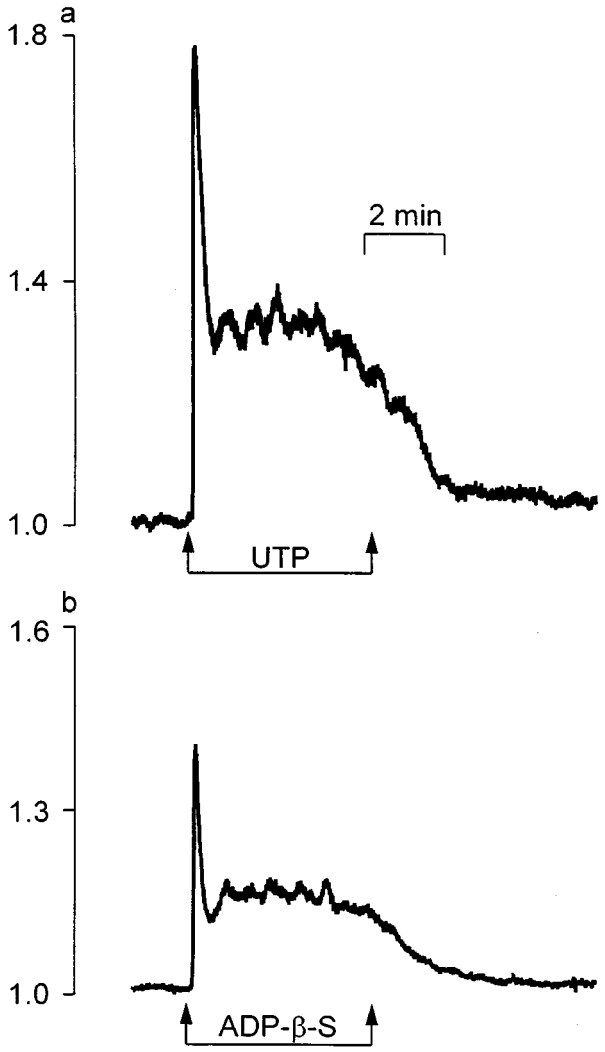

Figure 1 Response to a single application of 100 μM UTP (a) or 100 μM ADP-β-S. (b) Data were recorded from single cells and, in both instances, essentially identical responses obtained in seven experiments.

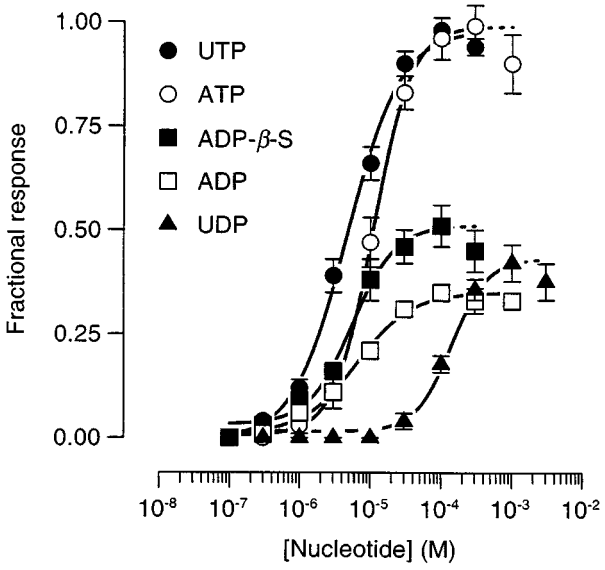

Figure 2 Responses to ATP ($n=8$), UTP ($n=15$) ADP ($n=5$), ADP-β-S ($n=6$) and UDP ($n=5$) (0.1 μM – 10 mM) were expressed as fractions of the response elicited by 100 μM ATP and these data are plotted (means $\pm$ s.e.mean) against the concentration of nucleotide used. The solid lines are sigmoid curves fitted to the pooled data.

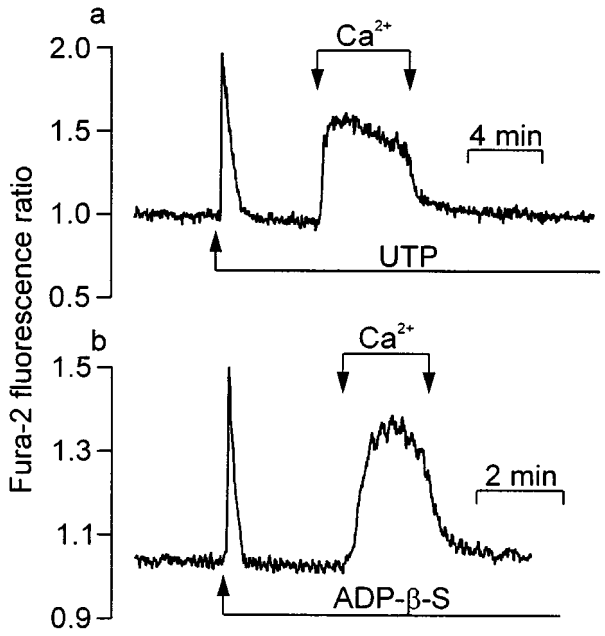

Figure 3 Role of internal and external Ca^{2+} in the responses to UTP (a) and ADP-β-S (b). Each panel shows a record from a single experiment although, for both nucleotides, essentially identical data were obtained in four experiments. Cells were initially superfused with Ca^{2+}-free saline and exposed to the appropriate nucleotide (100 μM) before external Ca^{2+} was restored to 1 mM for the indicated period.

Effects of pertussis toxin upon the responses to UTP and ADP-β-S

The data presented in Figure 4 show that pertussis toxin reduced the maximal response to UTP by $\sim$50% but had no effect upon the nucleotide's potency (E_{50} values: control, 1.3 ± 0.1 μM, pertussis toxin-treated 0.9 ± 0.47 μM). The toxin did not, however, have any effect upon the responses to ADP-β-S (Figure 4b, EC_{50} values: control, 4.1 ± 0.8 μM, pertussis toxin-treated, 3.4 ± 0.3 μM).

Effects of repeated stimulation with UTP or ADP-β-S

Repeatedly stimulating the cells with 1 min pulses of 100 μM UTP, delivered at intervals of 6 min, elicited a series of $[Ca^{2+}]_i$-transients. There was, however, a progressive decline in the magnitude of each successive response (Figure 5a) and analysis showed that the response to UTP normally decayed monotonically ($t_{1/2}=17.2\pm3.2$ min) towards a value (0.10 ± 0.07 ratio units) that was $\sim$15% of the response evoked by the initial stimulus. The cells lost sensitivity to UTP more rapidly (4.6 ± 0.6 min, $P<0.001$) in the presence of 16.2 nM TPA although the responses decayed towards a limiting value (0.12 ± 0.02 ratio units) essentially identical to that seen under control conditions. Conversely, this loss of sensitivity was slowed ($t_{1/2}=1.9\pm0.4$ h, $P<0.001$) by 0.1 μM staurosporine (Figure 6a). In further experiments ($n=6$), cells were again stimulated with pulses of 100 μM UTP. The peak rise in the Fura-2 fluorescence ratio elicited by the first pulse was 0.80 ± 0.04 ratio units and, thereafter, each stimulus elicited a steadily smaller response until the third stimulus increased the fluorescence ratio by only 0.47 ± 0.03 units. This confirms ($P<0.001$, paired t-test) that the cells become desensitized to UTP during repeated stimulation. In these experiments, however, the cells were exposed to 0.1 μM

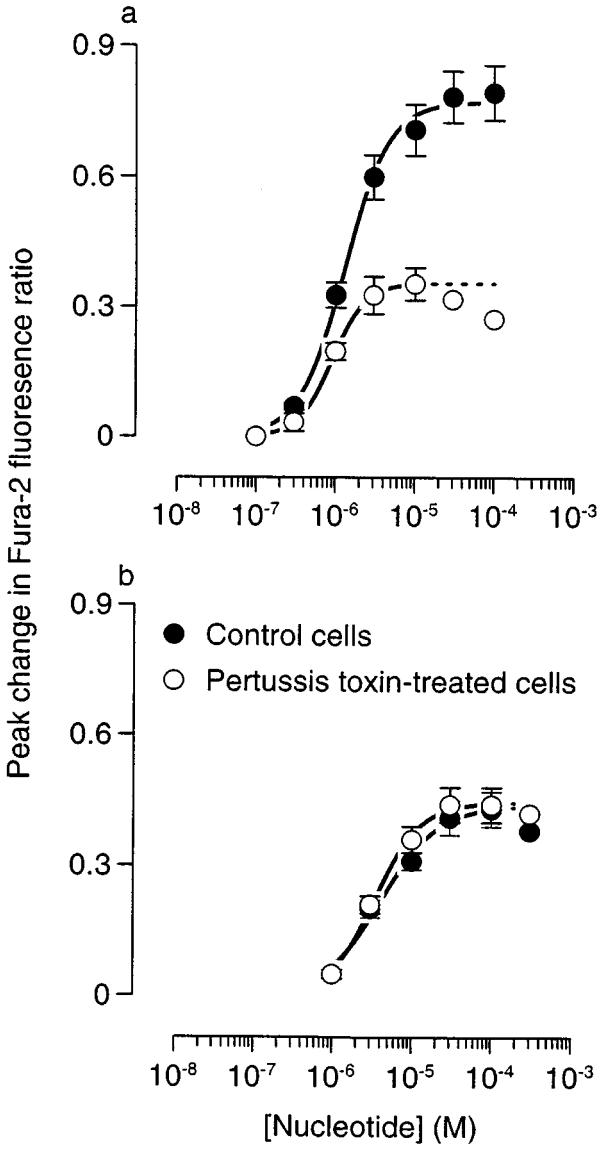

Figure 4 (a) Control ($n=6$) and pertussis toxin-treated ($n=6$) cells at identical passage were exposed to a series of 30 s pulses of increasing concentrations of UTP and the magnitude of the resultant increases in Fura-2 fluorescence ratio are plotted against the concentration of nucleotide used. (b) Data from directly analogous experiments in which control ($n=8$) and pertussis toxin-treated ($n=8$) cells were stimulated with ADP-β-S. The solid lines show sigmoid curves fitted to the experimental data. Analysis showed that pertussis toxin significantly ($P<0.05$, Student's t-test) reduced the magnitude of the responses to all UTP concentrations above 0.1 μM, but had no effect upon the responses to ADP-β-S.

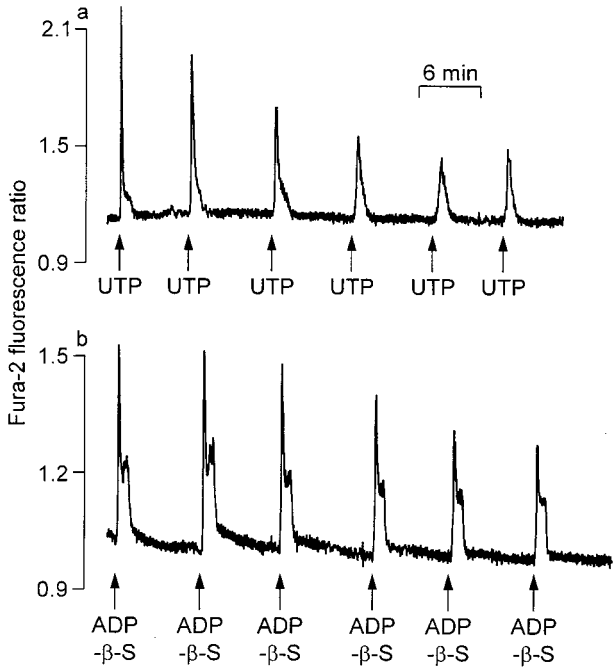

Figure 5 (a) A typical trace showing the $[Ca^{2+}]_i$-transients elicited by repeatedly stimulating single cells with a series of 1 min pulses of 100 μM UTP. Essentially identical data obtained in 17 experiments. (b) Data recorded during a directly analogous experiment in which single cells were repeatedly stimulated with 100 μM ADP-β-S (b). Essentially identical responses were obtained in six experiments.

staurosporine from immediately after the third pulse was delivered. This drug elicited a progressive rise in the magnitude of each successive response until, by the sixth pulse the rise in Fura-2 fluorescence did not differ significantly (0.65 ± 0.03 ratio units) from the initial control response. Staurosporine can thus slow the desensitization to UTP and reverses the loss of sensitivity seen in cells that have been repeatedly stimulated with this nucleotide.

Repeated stimulation with ADP-β-S was also associated with desensitization (Figure 5b) although analysis of the data showed that this process was slower ($t_{1/2}=36.8\pm4.5$ min) than seen in UTP-stimulated cells ($P<0.005$). This loss of sensitivity to ADP-β-S was, however, essentially abolished ($t_{1/2}>3$ h) by staurosporine ($P<0.01$) but greatly accelerated ($t_{1/2}=4.8\pm0.5$ min) by TPA ($P<0.001$) (Figure 6b). Interestingly, TPA caused essentially complete ($99\pm1\%$, $n=6$) loss of sensitivity to ADP-β-S (Figure 6b) whereas there was a clear, TPA-resistant component to the response to UTP (Figure 6a).

Desensitization in pertussis toxin-treated cells

Experiments in which pertussis toxin-treated cells were repeatedly stimulated with UTP showed that each nucleotide pulse elicited an essentially identical response (Figure 6a) and analysis confirmed that the loss of sensitivity seen in control cells was abolished ($P>0.001$) by this toxin ($t_{1/2}>3$ h). Moreover, although staurosporine had no effect on the responses seen during repeated stimulation with UTP ($t_{1/2}>3$ h, Figure 6a), TPA ($P<0.001$) elicited a rapidly developing ($t_{1/2}=5.5\pm0.1$ min) and essentially complete loss of sensitivity (Figure 6a). Pertussis toxin did not have any statistically significant effect upon the pattern of desensitization seen in ADP-β-S-stimulated cells (Figure 6b), either under control conditions ($t_{1/2}=54.3\pm16.7$ min) or in the presence of staurosporine ($t_{1/2}>3$ h) or TPA ($t_{1/2}=5.0\pm0.6$ min).

Discussion

Receptors for extracellular nucleotides fall into two broad groups: P2X receptors that act as cation channels gated by extracellular nucleotides and P2Y receptors that are coupled to their intracellular effectors *via* heterotrimeric G proteins. Moreover, each receptor category can be further subdivided

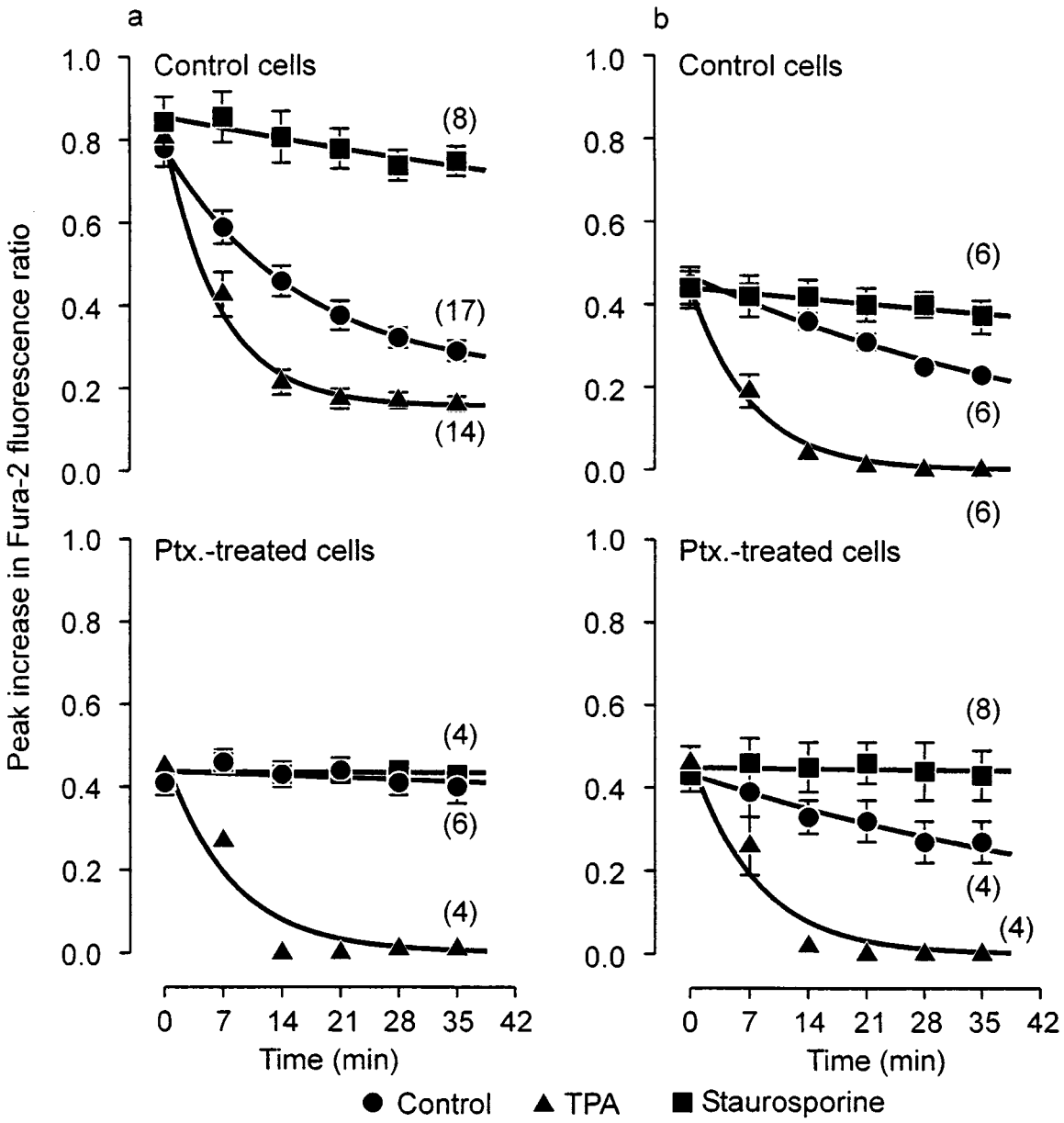

Figure 6 In each experiment, control (upper panels) or pertussis toxin treated cells were repeatedly stimulated with pulses (1 min) of 100 μM UTP (a) or 100 μM ADP-β-S (b), and the first such stimulus was always delivered under control conditions. Control cells were superfused with the standard physiological salt solution throughout the remainder of the experiment whilst the experimental cells were exposed to either 0.1 μM staurosporine or 16.2 nM TPA from 3 min before the delivery of the second nucleotide pulse. The responses to each nucleotide pulse are expressed as fractions of the response elicited by the initial, control stimulus and these data are plotted (mean$\pm$s.e.mean) against time. Values of *n* are presented in parenthesis.

into several pharmacologically distinct subclasses (Dubyak & El-Moatassim, 1993; Valera *et al*., 1994; Fredholm *et al*., 1997; Burnstock, 1998). There are, therefore, a large number of mechanisms that allow nucleotides to contribute to the control of vascular function although it is generally assumed to be receptors belonging to the $P2X_1$ subclass that allow vascular smooth muscle cells to respond to ATP (Benham & Tsien, 1987; Benham, 1989; Boarder & Hourani, 1998; Burnstock, 1998). However, the cultured aortic myocytes used in the present study did not respond to the $P2X_1$ agonist α,β-me-ATP, confirming that this receptor subclass is not expressed by smooth muscle cells maintained in culture (see e.g. Droogmans *et al*., 1991; Kalthof *et al*., 1993; Pacaud *et al*., 1995).

The rank order of potency amongst the effective nucleotides was UTP > ADP-β-S > ADP > ATP > UDP and this did not coincide with the pharmacological profile of any known P2 receptor subtype (Dubyak & El-Moatassim, 1993; Nicholas *et al*, 1996; Communi & Boeymans, 1997; Boarder & Hourani, 1998; Burnstock, 1998). The fact that UDP was effective only at concentrations >30 μM suggested strongly that $P2Y_6$ receptors were not present (Communi & Boeymans, 1997) and the complete lack of sensitivity to adenosine and AMP excludes a role for Ca^{2+}-mobilizing A_2 receptors (Burnstock, 1998). However, the $P2Y_1$ receptor agonist ADP-β-S consistently increased $[Ca^{2+}]_i$ suggesting strongly that these receptors were present. Although $P2Y_1$ receptors are insensitive to pyrimidine nucleotides, the cultured cells consistently responded to UTP and so a second population of receptors must underlie the responses to this nucleotide. At least two such receptor subtypes, $P2Y_2$ and $P2Y_4$, have been identified

although they have different pharmacological properties. $P2Y_2$ receptors are thus insensitive to ADP and UDP whilst these compounds act as partial $P2Y_4$ receptor agonists (Parr *et al*., 1994; Communi *et al*., 1995; Rice *et al*., 1995; Nicholas *et al*., 1996; Communi & Boeymans, 1997; Boarder & Hourani, 1998). However, in rat tissues, ATP and UTP activate both $P2Y_2$ and $P2Y_4$ receptors with essentially identical efficacy and potency (Bodganov *et al*., 1998) and so the responses to UTP can be attributed to expression of $P2Y_2$ and/or $P2Y_4$ receptors.

In subsequent experiments cells were therefore stimulated with either ADP-β-S or UTP, activating the $P2Y_1$ and $P2Y_{2/4}$ subclasses respectively. This is consistent with data presented by others (e.g. Pacaud *et al*., 1995; Malam-Souley *et al*., 1996; Strøbæk *et al*., 1996) although we cannot exclude the possibility that other unidentified receptors may contribute to the present responses.

Signal transduction pathways underlying the Ca^{2+} signals

UTP and ADP-β-S both mobilized Ca^{2+} from cytoplasmic stores which supports the view that these nucleotides act upon G protein-coupled receptors that allow control over PI-PLC (Sternweis & Smrcka, 1992; Berridge, 1993; Dubyak & El-Moatassim, 1993; Nicholas *et al*., 1996; Communi & Boeymans, 1997; Boarder & Hourani, 1998; Burnstock, 1998). Although two G protein families, $G_{q/11}$ and $G_{i2/3}$, permit such control, they do so by different mechanisms. The α subunits derived from $G_{q/11}$ ($\alpha_{q/11}$) thus activate the β_1 isoform of PI-PLC (PI-PLC-β_1) whilst the $\beta\gamma$ subunits from $G_{i2/3}$ control PI-PLC-$\beta_{2/3}$ activity (Camps *et al*., 1992; Katz *et al*., 1992; Sternweis & Smrcka, 1992; Berridge, 1993). It is therefore interesting that the maximal response to UTP was greater than that evoked by ADP-β-S, and that this additional effect of UTP was abolished by pertussis toxin, which blocks signalling *via* all known G_i families as well as *via* G_o. However, pertussis toxin did not effect the response to ADP-β-S. It thus appears that the responses to $P2Y_{2/4}$ receptor agonists are mediated by $G_{q/11}$ and $G_{i/o}$ whilst $P2Y_1$ receptors can only transmit signals across the plasma membrane *via* $G_{q/11}$ (Cockcroft & Stutchfield, 1989; Dubyak & El-Moatassim, 1993; Purkiss *et al*., 1994; Wilson *et al*., 1996).

Desensitization during repeated stimulation

The lipid product of PIP_2 hydrolysis, a diacylglycerol (DAG), remains in the plasma membrane and allosterically modulates the activity of protein kinase C (PKC), a Ca^{2+}- and phospholipid-dependent enzyme that controls many aspects of cell metabolism (Nishizuka, 1988). There is evidence that the rise in PKC activity that occurs during stimulation with PI-PLC-coupled agonists exerts negative feedback over the receptor-mediated activation of PI-PLC. Increased activity of PKC can, therefore, cause loss of sensitivity during prolonged or repeated stimulation (Llano & Marty, 1987; Maruyama, 1989; Ko *et al*., 1994). The present study showed clearly that stimulation with UTP or ADP-β-S could elicit desensitization and experiments using TPA and staurosporine, were consistent with the hypothesis that this may be due to increased activity of this enzyme (Llano & Marty, 1987; Maruyama, 1989; Ko *et al*., 1994). However, staurosporine is relatively non-selective, and so other explanations are possible.

Desensitization in pertussis toxin-treated cells

In pertussis toxin-treated cells, TPA elicited complete loss of sensitivity to UTP but there was always a TPA-resistant component to the control response. However, the responses to ADP-β-S, which were insensitive to pertussis toxin, were abolished by TPA. The pertussis toxin-resistant part of the response to UTP, and the entire response to ADP-β-s, thus appear to be subject to PKC-dependent down regulation whilst the pertussis toxin-sensitive part of the response to UTP does not. These data thus suggest that transmembrane signaling *via* $G_{q/11}$, but not *via* $G_{i/o}$, can be down regulated by PKC. Studies of P2Y receptor-mediated signalling in aortic endothelial cells (Purkiss *et al*., 1994) are interesting in this context. In these cells, responses to $P2Y_1$ receptor agonists were unaffected by pertussis toxin and subject to essentially complete down regulation by TPA, which accords the present data. However, when compared to smooth muscle cells, the endothelial $P2Y_{2/4}$ receptors appeared more sensitive to pertussis toxin but essentially insensitive to TPA (Purkiss *et al*., 1994). These data suggest that the $P2Y_{2/4}$ receptors in the endothelium are strictly coupled to PI-PLC *via* $G_{i/o}$, but that the equivalent receptors in cultured smooth muscle cells can also transmit signals across the plasma membrane *via* $G_{q/11}$.

As well as reducing the maximal response to UTP, pertussis toxin abolished the desensitization seen during repeated stimulation with this nucleotide. This suggests that the activation of PKC, which appears to underlie the desensitization in control cells, is dependent upon the ability to signal *via* $G_{i/o}$. Initially, we found this difficult to explain as we had assumed that IP_3 and DAG must both originate from the PI-PLC-mediated hydrolysis of PIP_2. However, in the endothelium, $P2Y_{2/4}$ receptor agonists elicit the release of DAG from both PIP_2 and phosphatidyl choline (Billah & Anthes, 1990; Purkiss *et al*., 1993). Interestingly, in A431 epithelial cells, pertussis toxin blocks the formation of DAG from PC but not from PIP_2 (Wang *et al*., 1991). It is therefore possible that pertussis toxin may inhibit the receptor-mediated activation of PKC in the smooth muscle cells, which may explain why this toxin blocked the desensitization seen during repeated stimulation with UTP.

The signal transduction pathways that can be controlled *via* $P2Y_1$ and $P2Y_{2/4}$ receptors in cultured smooth muscle cells are thus differentially sensitive to pertussis toxin and PKC-dependent desensitization. The subtle differences between the pathways controlled by these closely related receptors may be important in understanding the complex effects of nucleotides upon normal and diseased blood vessels.

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